

TYPING OF HUMAN ENTEROVIRUSES

FIELD OF THE INVENTION

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The present invention relates to methods of detecting the presence, and of establishing the serotype, or serovar, of an enterovirus that may be present in a clinical sample or a biological sample, as well as to a kit that includes primers that may be used in the methods. The methods include amplification of viral RNA, and sequencing of the resulting amplicons.

BACKGROUND OF THE INVENTION

10 Enteroviruses constitute a broad range of pathogens etiologically responsible for a wide range of diseases in humans, as well as in other animals. The genus *Enterovirus* is a member of the family *Picornaviridae*. As the family name indicates, enteroviruses are small RNA viruses; they contain positive single stranded RNA as the genome. Five groups are found within the enteroviruses: coxsackievirus A (CA), coxsackievirus B (CB), echovirus (E), and numbered enteroviruses (EV), as well as
15 poliovirus (PV). There are 66 serotypes currently classified among the human enteroviruses, although two serotypes, E22 and E23, are to be reclassified in a different genus.

The viral genome is shown schematically in Figure 1. The single stranded RNA comprises a 5' nontranslated region (single line), which is followed by an open
20 reading frame coding for a polyprotein precursor of Mr 240-250 x 10³ Da (boxed portion), followed by a 3' noncoding sequence and a poly (A) tract (single line). In the polyprotein, the sequence of gene products begins 1A, 1B, 1C, 1D, and 2A. 1A through 1D are, respectively, the structural proteins VP4, VP2, VP3, and VP1 of the viral capsid; VP1 is followed in the open reading frame by a nonstructural protein 2A.

25 The various members of the human enteroviruses cause a wide range of symptoms, syndromes and diseases. These include acute benign pericarditis, acute flaccid paralysis, acute hemorrhagic conjunctivitis, aseptic meningitis, various exanthemas, carditis, croup, encephalitis, enanthema, gastrointestinal disease,

hepatitis, hand-foot-and-mouth disease, various respiratory diseases, myocarditis, neonatal disease including multi-organ failure, pericarditis, pleurodynia, rash, and undifferentiated fever. In general, the syndromes are not correlated with particular enterovirus serotypes, nor does a serotype specifically correlate with a particular disease, although in certain cases serotypes do correlate with particular diseases.

Enteroviruses are responsible for large numbers of infections. There may be between 30 million to 50 million illnesses that are ascribable to enteroviruses each year in the United States (CDC; MMWR 46:748-750; Strikas et al. J. Infect. Dis. 146:346-351 (1986); Rotbart in Human Enterovirus Infections, H. A. Rotbart (ed.) ASM Press, Washington, DC, pp. 401-418 (1995)). After rhinoviruses, enteroviruses are the most common viral infection in humans. Enteroviral infections lead to 30,000 to 50,000 hospitalizations each year for aseptic meningitis, myocarditis, encephalitis, acute hemorrhagic conjunctivitis, nonspecific febrile illnesses, and upper respiratory infections (Melnick, Biologicals 21:305-309 (1993); Morens et al. in Human Enterovirus Infections, H. A. Rotbart (ed.) ASM Press, Washington, DC, pp. 3-23 (1995); Melnick in Fields Virology (B. N. Fields et al. (eds.) 3rd ed., Lippincott-Raven Publishers, Philadelphia, pp. 655-712 (1996)). Enteroviruses are also implicated in acute flaccid paralysis in animal models, as well as in dilated cardiomyopathy. The six serotypes of coxsackie B viruses are implicated in a variety of clinical diseases, such as meningitis, myocarditis and severe neonatal disease. Recently, enterovirus infection has been linked to chronic fatigue syndrome (Clements et al., J. Med. Virol. 45:156-161 (1995)).

Poliovirus is also an enterovirus that infects humans. Three serotypes, PV1, PV2, and PV3 are known. A nonenteroviral picornavirus that also afflicts humans is human rhinovirus (HRV), responsible for many common cold infections; several serotypes have been identified. Additionally, picornaviruses affect mammals other than humans, including viruses such as bovine enterovirus (BEV) and simian picornavirus (SPV).

It is important to identify the serotype of an enterovirus infection in a subject. Knowledge of the serotype can provide useful guidance to a physician in determining

a course of treatment of the disease in the subject. For example, the appropriately identified immune globulin having a sufficient titer may be administered to immunocompromised patients. Furthermore, an antiviral drug such as Pleconaril (Viropharma) may differ in its relative efficacy against different serotypes.

5 Additionally, an understanding of the geographic and chronological development of an enterovirus infection in a population can influence preventive measures among the members of the population to minimize the spread of the disease. Furthermore, it is useful from a broader perspective to track the incidence and distribution of an enterovirus disease from an epidemiological point of view. In earlier practice, it was
10 found that the various serotypes could be grown in different cell culture hosts, and in different animal model hosts. In the animal hosts, furthermore, different symptomology also provided typing information. These classical assays provide ways of distinguishing the serotypes. Nevertheless, some enterovirus serotypes, especially in the coxsackievirus A group, do not grow in cell culture. It has been observed that
15 25% to 35% of patient specimens are not identified by cell culture for a variety of reasons (Rotbart, 1995). Furthermore, such culturing and classification procedures are costly, time-consuming, subject to experimental variation, and not amenable to repetitive or extensive application in the field.

The serotypes of non-polio enteroviruses have been identified during the past
20 several decades using classical immunological neutralization assays based on a panel of specific antibodies. Application of such a determination to a clinical sample is generally impractical and inconvenient. Although a number of neutralization sites have been localized to the VP1 protein of enteroviral particles, the exact identity of the epitopes responsible for serotype specificity remain unknown; VP2 and VP3 may
25 also contain specific neutralizing epitopes. Serotyping has generally been carried out using intersecting pools of antisera, the Lim and Benyesh-Melnick (LBM) pools, which were originally defined in 1960 (Lim et al., J. Immunol. 84:309-317 (1960)). The antiserum pools currently distributed by the World Health Organization cover 42 serotypes in 8 pools (Melnick et al., Bull. WHO 48:263-268 (1973)). Analysis of the
30 neutralization pattern affords an identification of serotype. (See Rotbart, 1995).

Clearly, this is a cumbersome and painstaking process. Additionally, the supply of the antisera is limited or difficult to maintain. Problems in serotyping more recent isolates have been ascribed to pronounced intratypic antigenic variation (Melnick, Enteroviruses: polioviruses, coxsackie viruses, echoviruses, and newer enteroviruses.

5 In Fields Virology (Fields et al., (Eds.) 3rd Ed., Lippincott-Raven Publishers, Philadelphia, 1996, pp. 655-712; Melnick et al., Bull. W.H.O. 63:453-550 (1985); Wigand et al., Arch. Ges. Virusforsch. 12:29-41 (1962); Wenner et al., Am J. Epidemiol. 85:240-249 (1967); Duncan, Arch. Ges. Virusforsch. 25:93-104 (1968)). This has been explained by pointing out that enteroviruses, being RNA viruses, 10 undergo spontaneous mutation at a very high rate. This can lead to antigen drift, with the potential of producing antigenic variants such that a neutralization assay would produce a false negative result. For example, escape mutants in picornaviruses are discussed in detail in Mateu (Virus Res. 38:1-24 (1995)). For all these reasons there is a need to supplant neutralization assays for serotyping non-polio enteroviruses.

15 More recently assays based on nucleic acid detection have been developed. Probe hybridization assays directed either to RNA or to cDNA have been used to detect non-polio enteroviruses (Rotbart et al., Mol. Cell. Probes 2:65-73 (1988); Rotbart, J. Clin. Microbiol. 28:438-442 (1990); Chapman et al., J. Clin. Microbiol. 28: 843-850 (1990); Hyypia et al., J. Gen. Virol. 70:3261-3268 (1989); Olive et al. J. Gen. 20 Virol. 71:2141-2147 (1990); Gilmaker et al., J. Med. Virol. 38:54-61 (1992); Yang et al., Virus Res. 24:277-296 (1992); Zoll et al., J. Clin. Microbiol. 30:160-165 (1992); Muir et al., J. Clin. Micro. 31:31-38 (1993); Drebot et al., J. Med. Virol. 44:340-347 (1994); Rotbart et al., J. Clin. Microbiol. 32:2590-2592 (1994)). In the absence of nucleic acid sequence information for the non-polio enteroviruses, most of these 25 probes have targeted the highly conserved 5' non-coding region of the viral genomes. Additionally, RNA probes directed to the VP1 capsid gene have been used on a limited basis to identify some of the CBs and a few closely related CAs (Cova et al., J. Med. Virol. 24:11-18 (1988); Alksnis et al., Mol. Cell. Probes 3:103-108 (1989); Petitjean et al., J. Clin. Microbiol. 28:307-311 (1990)). More recently, 30 oligonucleotides having sequences based on the VP4-VP2 junction have been applied

as diagnostic and epidemiologic tools (Drebot et al., J. Med. Virol. 44:340-347 (1994); Arola et al., J. Clin. Microbiol. 34:313-318 (1996); Kim et al., Arch. Virol. 142:853-860 (1997); Oberste et al., Virus Res. 58:35-43 (1998)). The sequences in these regions, however, do not always correlate with serotype (Kopecka et al., Virus Res. 38:125-136 (1995); Arola et al., J. Clin. Microbiol. 34:313-318 (1996)).
Furthermore, sequences of only certain prototypes were available with which to compare and classify clinical samples (Arola et al., (1996)). A generic probe-based assay for nucleic acids in the presence of chaotropic agents is described in U.S. Patent 5,726,012. An assay for a target nucleic acid sequence wherein two separate probes are hybridized to the same strand of a nucleic acid, and then joined, for example by a polymerase activity, is disclosed in U.S. Patent 5,516,641.

Reverse transcription (RT) coupled with the polymerase chain reaction (PCR) (RT-PCR) has been developed using enterovirus universal primers or broadly selective primers. Such primers are intended to amplify nucleotide regions from a large number of enterovirus serotypes in one diagnosis. One set of primers (Rotbart, J. Clin. Microbiol. 28:438-442 (1990)) has been reported to amplify 60 of the 66 serotypes tested. (Among the nonreactive serotypes, two are atypical enteroviruses and may be reclassified.) A comparison of sequence identities of the various sets of universal primers with serotype sequences is given in Rotbart et al. (1995). Many of the universal primer sets are designed to amplify regions of the 5' untranslated region of the genome (see, for example, Drebot et al. (1994); Diedrich et al., J. Med. Virol. 46:148-152 (1995); Arola et al. (1996); Bailly et al., Virology 215:83-96 (1996); and U.S. Patent 5,075,212 to Rotbart). A comparison of base sequences in coxsackievirus B5 was reported for isolates from three different outbreaks of disease, based on amplicons obtained using primers in the VP1/2A region of the genome (Kopecka et al., (1995)). Variations in sequence occurred even within the same outbreak, and somewhat greater variations were found among isolates from the different outbreaks, and between serotypes. International application WO 98/14611 discloses degenerate primers directed to the VP1 gene, which, when used in certain defined pairs, provide PCR amplification of enterovirus nucleic acids. Use of the specific primer pairs

permits ascertaining whether a sample belongs to an enterovirus serotype, or to a small group of cognate serotypes, based on correlation of the pattern of the presence or absence of an amplicon with priming by the various primer pairs. This method does not rely on obtaining nucleotide sequences for accomplishing the serotyping.

5 Oberste et al. developed a database of homologous sequences for a portion of the VP2 gene of all 66 human enterovirus serotypes (Virus Res. 58:35-45 (1998a)). They found that the sequences of many antigenic variants failed to cluster with their respective prototype strains as determined by serotyping. This finding suggested that the portion of VP2 examined may not prove to be useful for consistent molecular
10 inference of serotype.

According to Holland et al. (J. Clin. Microbiol. 36:1588-1594 (1998)) neither cell culture growth, nor PCR can successfully type enterovirus infections. They report an alternative typing protocol based on polyacrylamide gel electrophoretic fingerprinting of whole virus radiolabeled proteins. However, the database of viral
15 protein profiles contains data for less than one-third of the known EV serotypes. Therefore its general applicability remains unknown.

In the case of poliovirus, U.S. Patents 5,585,477 and 5,691,134 to Kilpatrick disclose methods and oligonucleotide primers that are specific and sensitive for detecting all genotypes of poliovirus, as well as primers that are specific and sensitive
20 for distinguishing the three serotypes of poliovirus, and methods for detecting poliovirus and/or distinguishing among the serotypes based on the use of the disclosed primers. Additionally WO 98/14611 discloses an extensive set of degenerate oligonucleotide primers for use in detecting the presence or absence of a non-polio enterovirus in a sample and to identify non-polio enterovirus serotypes. The primers
25 are combined in pairs that detect various groupings of serotypes, and several amplification procedures are carried out in order to detect the presence or absence of an amplicon in each case. A pooled grid of the results provides information useful in typing a non-polio enterovirus in a sample.

In summary, immunological methods for serotyping enteroviral infections are
30 cumbersome and time consuming. They rely on an antigen-antibody reaction between

antiserum pools established more than two decades ago, and whose supply may become limited. As explained, for example in Mateu (1995), antigen drift among RNA viruses such as the enteroviruses leads to a high probability that escape mutants will arise, and thereby escape not only serotyping, but perhaps detection as well. A second classical approach, cell culture coupled with whole animal host growth and use of antisera for typing, is extremely cumbersome, expensive, and labor-intensive. Modern molecular biological methods similarly have important deficiencies as currently implemented. Probe assays generally tend to lack sensitivity. Furthermore, a probe directed to a conserved region, such as the 5' non-coding region of the non-polio enteroviruses, lacks specificity, and so cannot be readily applied in typing a viral infection. RT-PCR has been implemented as a generic enteroviral diagnostic assay. In general, these assays fail to implement serotype-specific detection, so that typing is not currently available using RT-PCR. Holland et al. (1998) state that all typing methods in use or then currently under development are limited by virtue of the large number of different enteroviral serotypes, and as a consequence, the need for virus-specific reagents that would discriminate among them.

For these reasons, there remains a need for a typing procedure that avoids the necessity of infecting live animals, animal tissue homogenates, or cell cultures. There further remains a need to implement a nucleic acid-based enteroviral typing procedure that optimizes the specificity required for a typing protocol. There additionally persists a need for a typing procedure that avoids a requirement for a plethora of reagents directed toward the specificity of the various serotypes. There still further remains the need for an enteroviral typing procedure that does not require extended periods of time or complicated procedures to carry out. Thus, there remains a need for an operationally elegant and efficient typing procedure that utilizes the specificity that resides, for example, in the VP1 region. The present invention recognizes these needs, and addresses them.

SUMMARY OF THE INVENTION

As noted above, the determinants of serotype identity are understood to reside primarily in VP1. This amino acid sequence specificity should be reflected in the corresponding VP1 gene sequences. The present invention discloses a method, based on reverse transcription and amplification of a characteristic enteroviral nucleic acid segment, for detecting the presence of an enterovirus in a clinical sample. The method includes the steps of

- (i) obtaining a clinical sample from a subject;
- (ii) purifying RNA contained in the sample;
- (iii) reverse transcribing the RNA with primers effective to reverse transcribe enteroviral RNA to provide a cDNA;
- (iv) contacting at least a portion of the cDNA with
 - (a) a composition that promotes amplification of a nucleic acid and
 - (b) an oligonucleotide mixture wherein the mixture comprises at least one oligonucleotide that hybridizes to a highly conserved sequence of the sense strand of an enterovirus nucleic acid and at least one oligonucleotide that hybridizes to a highly conserved sequence of the antisense strand of an enterovirus nucleic acid, thereby providing an amplification mixture, such that, upon hybridizing, the oligonucleotides direct amplification of at least a portion of the nucleotide sequence of the VP1 gene of the enterovirus genome;
- (v) carrying out an amplification procedure on the amplification mixture, such that, if an enterovirus is present in the sample, an enterovirus amplicon is produced whose sequence includes a nucleotide sequence of at least a portion of the VP1 region of the enterovirus genome; and
- (vi) detecting whether the amplicon is present.

The presence of the amplicon, of course, indicates that an enterovirus is present in the sample.

In important embodiments of the method, the highly conserved sequences occur within the VP1 gene or within about 100 nucleotides from a terminus of the

VP1 gene. Advantageously, at least one oligonucleotide of the mixture includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:80 or SEQ ID NO:81, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding a motif given by SEQ ID NO:82. Still more advantageously, the oligonucleotide mixture includes an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:3, and at least one oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:4, or an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:9. In a highly advantageous embodiment, the sequences of these three oligonucleotides are given respectively by SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:9.

In a further important embodiment of the method of detection, at least one oligonucleotide of the mixture includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding a motif given by SEQ ID NO:86, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:83, SEQ ID NO:84, or SEQ ID NO:85. In a further important embodiment, the oligonucleotide mixture contains an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:19, an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:20, and an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:21. In a still more important embodiment, the oligonucleotide mixture contains an oligonucleotide whose sequence is given by SEQ ID NO:22, and at least one oligonucleotide chosen from among oligonucleotides whose sequences are given by SEQ ID NOs:19, 20, and 21.

In further significant embodiments of the method, the amplification procedure includes a polymerase chain reaction, and the sample is obtained from among whole

blood or a fraction thereof, a bronchial wash, cerebrospinal fluid, an eye swab, a conjunctival swab, a swab or scraping from a lesion, a nasopharyngeal swab, an oral or buccal swab, pericardial fluid, a rectal swab, serum, sputum, saliva, stool, a stool extract, a throat swab, urine, brain tissue, heart tissue, intestinal tissue, kidney tissue, liver tissue, lung tissue, pancreas tissue, spinal cord tissue, skin tissue, spleen tissue, thymus tissue, cells from a tissue culture, a supernatant from a tissue culture, and tissue from an experimentally infected animal. In still other significant embodiments, the detection is carried out by a procedure chosen from among gel electrophoresis and visualization of amplicons contained in a resulting gel, capillary electrophoresis and detection of the emerging amplicon, probing for the presence of the amplicon using a labeled probe, and labeling a PCR primer employed in the method and detecting the label.

The invention additionally discloses a method for typing an enterovirus in a clinical sample that includes the steps of

- (i) obtaining a clinical sample from a subject;
- (ii) purifying RNA contained in the sample;
- (iii) reverse transcribing the RNA with primers effective to reverse transcribe enteroviral RNA to provide a cDNA;
- (iv) contacting at least a portion of the cDNA with
 - (a) a composition that promotes amplification of a nucleic acid and
 - (b) an oligonucleotide mixture wherein the mixture comprises at least one oligonucleotide that hybridizes to a highly conserved sequence of the sense strand of an enterovirus nucleic acid and at least one oligonucleotide that hybridizes to a highly conserved sequence of the antisense strand of an enterovirus nucleic acid, thereby providing an amplification mixture, such that, upon hybridizing, the oligonucleotides direct amplification of at least a portion of the nucleotide sequence of the VP1 gene of the enterovirus genome;
- (v) carrying out an amplification procedure on the amplification mixture, such that, if an enterovirus is present in the sample, an enterovirus sample amplicon

is produced whose sequence includes a nucleotide sequence of at least a portion of the VP1 region of the enterovirus genome;

(vi) determining that the sample amplicon is present;

(vii) determining at least a partial nucleotide sequence of the sample amplicon;

(viii) providing a database consisting of prototypical nucleotide sequences, wherein each prototypical sequence is the sequence of a standard amplicon obtained from a member of a set of prototypical enterovirus serotypes by carrying out the procedure of steps (ii) through (v) on each prototypical enterovirus serotype, wherein each prototypical sequence comprises at least a portion of the sequence of the VP1 gene, and wherein the sequence of each prototypical VP1 gene is different from the sequence of every other prototypical VP1 gene in the database;

(ix) comparing the sequence of the sample amplicon with each prototypical sequence in the database; and

(x) identifying the prototypical sequence that has the highest extent of identity to the sequence of the sample amplicon, thereby providing an identified serotype;

wherein the type of the sample is the serotype of the identified serotype.

In important embodiments of this method, the highly conserved sequences occur within the VP1 gene or within about 100 nucleotides from a terminus of the VP1 gene. More importantly, at least one oligonucleotide of the mixture includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:80 or SEQ ID NO:81, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding a motif given by SEQ ID NO:82. In significant embodiments of the method, the oligonucleotide mixture includes an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:3, at least one oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:4 or an oligonucleotide whose sequence contains, at the 3' end thereof,

the sequence given by SEQ ID NO:9. In a highly advantageous embodiment, the sequences of the oligonucleotides are given by SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:9.

In an additional important embodiment, at least one oligonucleotide of the mixture includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding a motif given by SEQ ID NO:86, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:83, SEQ ID NO:84, or SEQ ID NO:85. In a further important embodiment, the oligonucleotide mixture contains an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:19, an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:20, and an oligonucleotide whose sequence includes, at the 3' end thereof, the sequence given by SEQ ID NO:21. In a still more important embodiment, the oligonucleotide mixture contains an oligonucleotide whose sequence is given by SEQ ID NO:22, and at least one oligonucleotide chosen from among oligonucleotides whose sequences are given by SEQ ID NOs:19, 20, and 21.

In a further important aspect, the amplification procedure includes a polymerase chain reaction, and the resulting sample amplicon encompasses at least a portion of the nucleotide sequence for the VP1 gene of an enterovirus. The method furthermore importantly provides that the set of prototypical enterovirus serotypes comprises serotypes of coxsackie A viruses, coxsackie B viruses, echoviruses, and numbered enteroviruses. In advantageous aspects of the method, comparing the sequence of the sample amplicon with each sequence in the database employs a sequence alignment and comparison algorithm.

In further important aspects of the method, the sample is chosen from among whole blood or a fraction thereof, a bronchial wash, cerebrospinal fluid, an eye swab, a conjunctival swab, a swab or scraping from a lesion, a nasopharyngeal swab, an oral or buccal swab, pericardial fluid, a rectal swab, serum, sputum, saliva, stool, a stool

extract, a throat swab, urine, brain tissue, heart tissue, intestinal tissue, kidney tissue, liver tissue, lung tissue, pancreas tissue, spinal cord tissue, skin tissue, spleen tissue, thymus tissue, cells from a tissue culture, a supernatant from a tissue culture, and tissue from an experimentally infected animal.

5 The present invention further provides an oligonucleotide containing, at the 3' end thereof, a sequence that hybridizes to a nucleotide sequence encoding an amino acid motif chosen from among the sequences given by SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, and SEQ ID NO:86, or an oligonucleotide complementary to any of these oligonucleotides. In
10 an advantageous embodiment, the complete sequence of the oligonucleotide is a sequence that hybridizes to a sequence encoding a motif whose sequence is chosen from among SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, and SEQ ID NO:86, or is an oligonucleotide complementary to any of them.

15 In particularly important embodiments, such an oligonucleotide is one whose sequence contains, at the 3' end thereof, a sequence chosen from among the sequences given by SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, or an oligonucleotide whose sequence is complementary to any of these oligonucleotides. In still more important
20 embodiments, the sequence of the oligonucleotide consists of a sequence chosen from among SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:9, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, or an oligonucleotide that is complementary to any of them.

25 The present invention further discloses a mixture of oligonucleotides including at least two oligonucleotides, wherein at least one of the oligonucleotides hybridizes to a sense strand of a double stranded nucleic acid and at least one of the oligonucleotides hybridizes to an antisense strand of the nucleic acid. The nucleic acid to which the oligonucleotides hybridize encodes the VP1 gene of an enterovirus, and the oligonucleotides hybridize to sequences that are highly conserved among the
30 group of enteroviruses. The oligonucleotides, when hybridized to the nucleic acid, are

bound in the correct orientation on their respective strands to direct the synthesis of an amplicon encoding at least a portion of the VP1 protein of enteroviruses when they are employed in an amplification procedure using the nucleic acid.

In important embodiments of the mixture, each oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to the nucleic acid. In still more important embodiments, the highly conserved sequences occur within the VP1 gene or within about 100 nucleotides from a terminus of the VP1 gene. Advantageously, at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:80 or SEQ ID NO:81, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding an amino acid motif given by SEQ ID NO:82. Still more advantageously, the mixture includes an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:3, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:4, and an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:9. In a highly advantageous embodiment, the sequences of the oligonucleotides are given by SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:9.

In an important embodiment, at least one oligonucleotide of the mixture includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding a motif given by SEQ ID NO:86, and at least one oligonucleotide includes, at the 3' end thereof, a sequence that hybridizes to a sequence encoding the amino acid motif given by the sequences of either SEQ ID NO:83, SEQ ID NO:84, or SEQ ID NO:85.

In additional significant embodiments, the oligonucleotide mixture includes an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:19, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:20, and an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:21. In a still more significant

embodiment, the oligonucleotide mixture includes an oligonucleotide whose sequence is given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence is given by SEQ ID NO:19, an oligonucleotide whose sequence is given by SEQ ID NO:20, and an oligonucleotide whose sequence is given by SEQ ID NO:21.

The present invention additionally provides a kit for use in conducting the typing method that includes a mixture of oligonucleotides, the mixture containing an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:3, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:4, and an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:9. In important embodiments of the kit, the oligonucleotide sequences are given by SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:9.

In additional significant embodiments, the kit includes an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:19, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:20, and an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:21. In a still more significant embodiment, the oligonucleotide mixture includes an oligonucleotide whose sequence is given by SEQ ID NO:22, and at least one oligonucleotide chosen from among an oligonucleotide whose sequence is given by SEQ ID NO:19, an oligonucleotide whose sequence is given by SEQ ID NO:20, and an oligonucleotide whose sequence is given by SEQ ID NO:21.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the non-polio enterovirus genome.

Figure 2 illustrates RT-PCR amplification of all enterovirus prototype strains using primer pairs given by SEQ ID NOs:3 and 4, and by SEQ ID NOs: 3 and 9. PCR

products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. Panel A: Coxsackie A viruses, Coxsackie B viruses, and polioviruses amplified with primer pair given by SEQ ID NOs:3 and 4; Panel B: Coxsackie A viruses, Coxsackie B viruses, and polioviruses amplified with primer pair given by SEQ ID NOs: 3 and 9; Panel C: Echoviruses and numbered enteroviruses amplified with primer pair given by SEQ ID NOs: 3 and 4; Panel D: Echoviruses and numbered enteroviruses simplified with primer pair given by SEQ ID NOs: 3 and 9.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention advantageously provides methods for serotyping enteroviruses obtained from clinical samples. The methods are easily extended to human poliovirus, human picornaviruses such as human rhinovirus, and nonhuman picornaviruses such as bovine enterovirus and simian picornavirus. The procedures are easily and rapidly implemented using common laboratory procedures and instrumentation. They avoid the need for cumbersome, time-consuming and resource-intensive methods such as cell culture and/or host animal infection. They furthermore avoid reliance on prototypical antiserum pools which may fail to identify an enterovirus in a contemporary clinical sample because of antigen drift and escape from immunological reactivity. The methods of the present invention further advantageously permit identifying a serotype as being the most probable serotype even in the case of antigen drift, since nucleotide sequences are matched to provide a most probable serotype match, or, failing a unique match, a set of most probable serotype matches, even in the absence of a high extent of identity.

20 As used herein, the non-polio enteroviruses refer to the species/subgroups and serotypes, shown in Table 1, that are known in the field at the present time.

Table 1. Non-polio Enterovirus Species/Subgroups and Serotypes.

Species/Subgroup	Serotypes ^a
Coxsackievirus A	CA1 to CA22, CA24
Coxsackievirus B	CB1-CB6
Echovirus	E1-E7, E9, E11-E27, E29-
Enterovirus (Numbered)	EV68-EV71

(a). Serotypes CA-23, E-10, E-28, and EV-72 have been reclassified (Miller, Clin. Infect. Dis. 16:612-613 (1993)). E-8 has been reclassified (Committee on the Enteroviruses, Virology 16:501-504 (1962); Harris et al., J. Infect. Dis. 127:63-68 (1973)).

As used herein, a "clinical sample" or a "clinical isolate" relates to any sample obtained from a subject for use in carrying out the procedures of the present invention. In a principal aspect, the subject is suspected of suffering from a disease or syndrome that is at least partially caused by an enterovirus. The subject may also be an asymptomatic individual considered to be at risk of enterovirus infection. The sample may be a cellular sample such as a tissue sample, for example, a sample of lung tissue obtained as a biopsy or post-mortem, a fluid sample such as blood, saliva, sputum, urine, cerebrospinal fluid, or a swabbed sample obtained by swabbing a mucus membrane surface such as a nasal surface, a pharyngeal surface, a buccal surface, and the like, or it may be obtained from an excretion such as feces, or it may be obtained from other bodily tissues or body fluids commonly used in clinical diagnostic testing. In its broadest sense, a "clinical sample" or a "clinical isolate" as used herein is obtained from a human subject or a non-human mammalian subject, and is directed to suspected symptoms or syndromes ascribable to a picornavirus or enterovirus infection.

As used herein, purification of RNA as a step in the methods of the invention, in particular, as a step leading up to a RT-PCR procedure, relates to releasing RNA

from a latent or inaccessible form in a virion or a cell and allowing the RNA to become freely available. In such a state, it is suitable for effective amplification by reverse transcription and use of the polymerase chain reaction. Releasing RNA may include steps that achieve the disruption of virions containing viral RNA, as well as disruption of cells that may harbor such virions. Purification of RNA is generally carried out under conditions that rigorously and effectively exclude or inhibit any ribonuclease activity that may be present. Additionally, purification of RNA may include steps that achieve at least a partial separation of the RNA dissolved in an aqueous medium from other cellular or viral components, wherein such components may be either particulate or dissolved.

As used herein, "reverse transcription" or "RT" relates to a procedure catalyzed by an enzyme activity, reverse transcriptase, that synthesizes a cDNA from a single stranded RNA molecule, with the use of oligonucleotide primers having free 3'-hydroxyl groups. As used herein the term "polymerase chain reaction" or "PCR" relates to a procedure whereby a limited segment of a nucleic acid molecule, which frequently is a desired or targeted segment, is amplified repetitively to produce a large amount of DNA molecules which consist only of that segment. The procedure depends on repetition of a large number of priming and transcription cycles. In each cycle, two oligonucleotide primers bind to the segment, and define the limits of the segment. A primer-dependent DNA polymerase then transcribes, or replicates, the strands to which the primers have bound. Thus, in each cycle, the number of DNA duplexes is doubled.

As used herein the term "primer" or "oligonucleotide primer" relates to an oligonucleotide having a specific or desired nucleotide sequence which is complementary to a particular sequence on one of the strands of a DNA duplex. When the primer is caused to hybridize to the specific sequence in a DNA duplex to which it is complementary, it may serve as the priming position, or the initiation position, for the action of a primer-dependent DNA polymerase activity. The primer, once hybridized, acts to define the 5' end of the operation of the transcription activity of the polymerase on the duplex. Commonly in PCR, a specific pair of primers is

employed, wherein one of the primers hybridizes to one of the strands and the second primer hybridizes to the complementary strand. The primers hybridize in such an orientation that transcription, which proceeds in the direction from 5' to 3', is in the direction leading from each primer toward the site of hybridization of the other primer. After several rounds of hybridization and transcription the amplified DNA
5 produced is a segment having a defined length whose ends are defined by the sites to which the primers hybridize.

The oligonucleotide primers of the invention are intended for use in a RT-PCR-based amplification of a target segment of a nucleic acid from an enterovirus. Both RT and PCR rely on the action of a DNA polymerase activity to extend the new
10 DNA strands beyond the 3' termini of the primers. Since DNA polymerases extend a chain in the direction from 5' to 3', an oligonucleotide that contains sequences in addition to those nucleotides that hybridize to the target nucleic acid and serve as the primer must have the primer sequence at the 3' end of the oligonucleotide.
15 Additionally, any complements of the oligonucleotides contemplated in the invention must have the sequence complementary to the hybridizing sequence at the 5' end of the molecule such that action of a DNA polymerase will generate a primer oligonucleotide having its complementary sequence at its 3' end.

As used herein the terms "specific to" or "specific for" a target sequence, in relation to a nucleic acid sequence such as an oligonucleotide sequence, relate to a
20 nucleotide sequence that hybridizes, under conditions used in given experimental circumstances, to the target but does not hybridize under those circumstances to sequences that are not target sequences. Nucleotide sequences that are specific for a particular target, such as the enteroviral target sequences that are included in the
25 subject matter of the present invention, are those that include bases all of which are complementary to the corresponding base on the target.

Further as used herein, "specificity" of a nucleic acid sequence for a target sequence also encompasses nucleic acids and oligonucleotides having a small number of nucleotides which may not be complementary to the corresponding nucleotides of
30 the target sequence. Such sequences are still "specific" for the target sequence, as

used herein, as long as the extent of deviation from complementarity remains functionally of no consequence. In particular, such a sequence is "specific" for the target sequence as long as it hybridizes effectively to the target sequence but does not hybridize to any sequence that is not a target sequence, under the conditions used in given experimental circumstances.

As used herein, an "amplicon" relates to a double stranded nucleic acid segment having a defined size and sequence that results from an amplification procedure, such as a PCR procedure. The size of the amplicon is governed by the sites on the two strands of a nucleic acid duplex to which the primers bind. As explained in U.S. Patent 4,683,195, that segment of the product nucleic acid becomes the prevalent product of the amplification procedure after a small number of cycles of amplification.

As used herein, the terms "prototype", "prototypical sequence", "prototypical amplicon", and "prototypical enterovirus serotype" relate, insofar as the root "prototyp-" occurs in each of these terms, to the enterovirus serotypes which were used to establish the classical antisera defined against each serotype. These were originally obtained several decades ago, as described in Lim et al. (1960) and subsequently, for example, in Melnick et al. (Bull. Wld. Hlth. Org. 48:2163-268 (1973)), and Melnick et al. (1985). As used herein, these terms are distinguished from variants of a given prototypical serotype, wherein a variant represents a phenotype resulting from antigenic drift, such as a phenotype that may represent an escape mutant. Such variants may occur in the field among contemporary clinical isolates of enteroviruses.

As used herein, a "motif" relates to a short sequence of amino acid residues that is highly conserved among a family of proteins from different species or variants.

Developing a Database of Nucleotide Sequences Characteristic of the Prototypical Enteroviruses. In order to practice the methods of the present invention, a database of sequences characteristic of the prototypical enteroviruses is needed. In order to prepare such a database, a region of the enteroviral genome is selected that has within its nucleotide sequence sufficient variation among the

different serotypes that the sequence from each serotype may be considered to be unique. In the present invention, the VP1 region of the viral RNA was identified as having the requisite sequence uniqueness from one serotype to another. Among the entries in Table 2, below, direct comparison of results based on VP1 versus those obtained with VP2 for the following variants of the respective serotypes provided evidence that VP1 affords the selectivity required for this invention, whereas VP2 does not. The variants are CA24v strain EH24/70, E4 strain Du Toit, E4 strain Shropshire, E6 strain Charles, E6' strain Cox, E6" strain Burgess, E8 strain Bryson, E9 strain Barty, E11' strain Silva, E30 strain Frater, E30 strain Giles, E30 strain PR-17, E34 strain DN-19, PV1 strain Sabin, PV2 strain Sabin, and PV3 strain Sabin. Once such a region is identified, the nucleotide sequences from this region are determined for each virus among the set of prototypical serotypes. The serotype prototypes of interest in the present invention are listed in Tables 1 and 2; Table 2 includes entries for additional enteroviruses and picornaviruses as well. The viruses may be obtained from publicly available deposits made at the American Type Culture Collection (Rockville, MD).

Table 2. Enterovirus and Picornavirus VP1 Sequences Used in Establishing a Sequence Database

Serotype	Strain	GenBank Accession Number	SEQ ID NO:
CA1	Tompkins	AF081293	23
CA2	Fleetwood	L28146 (a)	
CA3	Olson	AF081294	24
CA4	High Point	AF081295	25
CA5	Swartz	AF081296	26
CA6	Gdula	AF081297	27
CA7	AB-IV	AF061298	28
CA8	Donovan	AF081299	29
CA9	Griggs	D00627 (b)	

Serotype	Strain	GenBank Accession Number	SEQ ID NO:
CA10	Kowalik	AF081300	30
CA11	Belgium-1	AF081301	31
CA12	Texas-12	AF081302	32
CA13	Flores	AF081303	33
CA14	G-14	AF081304	34
CA15	G-9	AF081305	35
CA16	G-10	U05876 (c)	
CA17	G-12	AF081306	36
CA18	G-13	AF081307	37
CA19	8663	AF081308	38
CA20	IH-35	AF081309	39
CA21	Kuykendall	D00538 (d)	
CA22	Chulman	AF081310	40
CA24	Joseph	AF081311	41
CA24v	EH24/70	D90457 (e)	
CB1	Conn-5	M16560 (f)	
CB2	Ohio-1	AF081312	42
CB3	Nancy	M16572 (g)	
CB4	JVB	D00149 (h)	
CB5	Faulkner	X67706 (i)	
CB6	Schmitt	AF081313	43
E1	Farouk	AF081314	44
E2	Cornelis	AF081315	45
E3	Morrissey	AF081316	46
E4	Pesacek	AF081317	47
E4	Du Toit	AF081318	48
E4	Shropshire	AF081319	49
E5	Noyce	AF081320	50
E6	Charles	U16283 (j)	

Serotype	Strain	GenBank Accession Number	SEQ ID NO:
E6	D'Amori	AF081321	51
E6'	Cox	AF081322	52
E6"	Burgess	AF081323	53
E7	Wallace	AF081324	54
E8	Bryson	AF081325	55
E9	Hill	X84981 (k)	
E9	Barty	X92886 (l)	
E11	Gregory	X80059 (m)	
E11'	Silva	AF081326	56
E12	Travis	X79047 (n)	
E13	Del Carmen	AF081327	57
E14	Tow	AF081328	58
E15	CII96-51	AF081329	59
E16	Harrington	X89545 (o)	
E17	CHHE-29	AF081330	60
E18	Metcalfe	AF081331	61
E19	Burke	AF081332	62
E20	JV-1	AF081333	63
E21	Farina	AF081334	64
E22	Harris	S45208 (o)	
E23	Williamson	AF055846 (p)	
E24	De Camp	AF081335	65
E25	JV-4	AF081336	66
E26	Coronel	AF081337	67
E27	Bacon	AF081338	68
E29	JV-10	AF081339	69
E30	Bastianni	AF081340	70
E30	Frater	AF081341	71
E30	Giles	AF081342	72

Serotype	Strain	GenBank Accession Number	SEQ ID NO:
E30	PR-17	AF081343	73
E31	Caldwell	AF081344	74
E32	PR-10	AF081345	75
E33	Toluca-3	AF081346	76
E34a	DN-19	AF081347	77
EV68	Fermon	AF081348	78
EV69	Toluca-1	AF081349	79
EV70	J670/71	D00820 (q)	
EV71	BrCr	U22521 (r)	
PV1	Mahoney	J02281(s)	
PV1	Sabin	V01150 (t)	
PV2	Lansing	M12197 (u)	
PV2	Sabin	X00595 (v)	
PV3	Leon	K01392 (w)	
PV3	Sabin	X00596 (v)	
BEV1	VG-5-27	D00214 (x)	
BEV2a	RM-2	X79369 (y)	
BEV2b	PS-87	X79368 (y)	
HRV3	Unknown	U60874	
PEV9	UKG/410/73	Y14459 (z)	
SVDV	H/3'76	D00435 (h)	
HRV1b	Unknown	D00239(dd)	
HRV2	Unknown	X02316 (aa)	
HRV3	Unknown	U60874	
HRV14	Unknown	K02121, X01087 (bb)	
HRV16	Unknown	L24917(ee)	
HRV89	41467 Gallo	M16248(ff)	
HAV	HM-175	M14707 (cc)	

Notes for Table 2:

- PEV, porcine enterovirus; SVDV, swine vesicular disease virus; HRV, human rhinovirus; HAV, hepatitis A virus.
- a) Pulli, T., et al., *Virology* 211:30-38 (1995).
 - b) Chang, K., et al., *J. Gen. Virol.* 70:3269-3280 (1989).
 - c) Poyry, T., et al., *Virology* 202:982-987 (1994).
 - d) Hughes, P.J., et al. *J. Gen. Virol.* 70:2943-2952 (1989).
 - e) Supanaranond, K., et al., *Virus. Genes* 6:149-158 (1992).
 - f) Iizuka, N., et al. *Virology* 156:64-73 (1987).
 - g) Lindberg, A. M., et al., *Virology* 156:50-63 (1987).
 - h) Jenkins, O., et al., *J. Gen. Virol.* 68:1835-1848 (1987).
 - i) Zhang, G., et al., *J. Gen. Virol.* 74:845-853 (1993).
 - j) Harris, L.F., et al., *J. Infect. Dis.* 127:63-68 (1973).
 - k) Zimmermann, H., et al., *Virus Res.* 39:311-319 (1995).
 - l) Zimmermann, H., et al., *Virus Genes* 12:149-154 (1996).
 - m) Dahllund, L., et al., *Virus Res.* 35:215-223 (1995).
 - n) Kraus, W., et al. *J. Virol.* 69:5853-5858 (1995).
 - o) Huttunen, P., et al., *J. Gen. Virol.* 77:715-725 (1996).
 - p) Oberste, M.S., et al., *Virus. Res.* 56:217-223 (1998).
 - q) Ryan, M.D., et al., *J. Gen. Virol.* 71:2291-2299 (1990).
 - r) Brown, B.A., et al., *Virus. Res.* 39:195-205 (1995).
 - s) Kitamura, N.B., et al., *Nature* 291:547-553 (1981); Racaniello, V.R., et al. *Proc. Natl. Acad. Sci. USA* 78:4887-4891 (1981).
 - t) Dorner, A.J., et al., *J. Virol.* 42:1017-1028 (1982); Emini, E. A., et al., *J. Virol.* 42:194-199 (1982); Nomoto, A., et al. *Proc. Natl. Acad. Sci. USA* 79:5793-5797 (1982).
 - u) La Monica, N., et al., *J. Virol.* 57:515-525 (1986).
 - v) Toyoda, H., et al. *J. Mol. Biol.* 174:561-585 (1984).
 - w) Stanway, G., et al. *Proc. Natl. Acad. Sci. USA* 81:1539-1543 (1984).
 - x) Earle, J. A., et al., *J. Gen. Virol.* 69:253-263 (1988).
 - y) McNally, R.M., et al., *Arch. Virol.* 139:287-299 (1994).
 - z) Peng, J., et al., Unpublished data.
 - aa) Skern, T., et al., *Nucl. Acids Res.* 13:2117-2126 (1985).
 - bb) Callaghan, P.L., et al., *Proc. Natl. Acad. Sci. USA* 82:732-736 (1985); Stanway, G., et al., *Nucl. Acids Res.* 12:7859-7875 (1984).
 - cc) Cohen, J. L., et al., *J. Virol.* 61:50-59 (1987).
 - dd) Hughes, P.J., et al., *J. gen. VFirol.* 69:49-58 (1988).
 - ee) Lee, W.M., et al., *Virus Genes* 9:177-181 (1995).
 - ff) Duechler, M., et al., *Proc Natl. Acad. Sci. USA* 84:2605-2609 (1987).

The virus specimens are used to infect any enterovirus-susceptible cell line in culture, including, by way of nonlimiting example, RD (human rhabdomyosarcoma) cells, HLF (human embryonic lung fibroblast) cells, LLC-MK₂ (monkey kidney) cells, or BGM (buffalo green monkey kidney) cells; alternatively, a tissue homogenate in

5 tissue culture medium may be prepared from mouse brain after infection of the mouse with the virus. In the case of cell cultures, the culture supernatant is used. In the case of the brain homogenate, the whole homogenate, after growth of the virus, is used.

Viral RNA is extracted from the growth media containing the enterovirus prototypes

by any method that releases the RNA from the virion and/or the cell components and provides a purified preparation of the RNA. By way of nonlimiting example, the RNA may be extracted using guanidinium isothiocyanate, such as the single-step isolation by acid guanidinium thiocyanate-phenol-chloroform extraction of
5 Chomczynski et al. (Anal. Biochem. 162:156-159 (1987)). Alternatively, the virion may be disrupted by a suitable detergent in the presence of proteases and/or inhibitors of ribonuclease activity. The RNA released from the virion is isolated or purified, using, for example, methods such as precipitation with an alcohol (e.g., ethyl alcohol or isopropyl alcohol) or banding in a suitable density gradient using an
10 ultracentrifuge.

The purified viral RNA is then subjected to a reverse transcription to prepare a cognate cDNA that encompasses the region of the genome chosen for discriminating between serotypes (i.e., the region encoding VP1). An advantageous way of achieving this is to use a set of random oligonucleotide primers in the reverse
15 transcription, such that certain of the primers in the set will hybridize to the RNA and yield one or more cDNA molecules from the virus encompassing the required serotype-specific nucleotide sequence. Alternatively, gene-specific primers based on a viral RNA-specific sequence from a suitable cDNA may be employed for reverse transcription. Subsequently, the cDNA is amplified using a suitable amplification
20 protocol. By way of nonlimiting example, a polymerase chain reaction (PCR) protocol may be employed for this purpose. PCR is described in operational detail in, for example, "Molecular Cloning: A Laboratory Manual," 2nd ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; "Current Protocols in Molecular Biology," Ausubel et al., John Wiley and Sons, New
25 York 1987 (updated quarterly); and "PCR Protocols: A Guide to Methods and Applications," Innis et al., Academic Press, San Diego, CA 1990; and in U.S. Patents 4,683,195; 4,683,202; 4,965,188; 5,578,467; 5,545,522; and 5,624,833, all of which are incorporated herein by reference.

For the PCR of the cDNA to yield an amplicon containing a sequence from the
30 VP1 region, primers such as those provided in Table 3 (SEQ ID NOs:1-22) may be

employed. In Table 3, nucleotide sequence positions are given relative to the sequence of poliovirus1-Mahoney (Kitamura, N.B., et al., Nature 291:547-553 (1981); Racaniello, V.R., et al. Proc. Natl. Acad. Sci. USA 78:4887-4891 (1981)).

Table 3. Primers Used for PCR Amplification of the VP1 Region of Enteroviruses

Primer	Sequence	Gene	Position	SEQ ID NO
008	GCRTGCAAGAYTTCTCWGT	VP3	2411-2430	1
009	NGCNCCDGAPPTTGNTGSCC	2A	3409-3391	2
011	GCICIGAYTGITGICCRAA	2A	3408-3389	3
012	ATGTAYGTICCCICGIGG	VP1	2951-2970	4
013	GGIGCRTTICCYTGIGTCCA	VP1	3051-3032	5
019	ACRTGICIGTYTGATIGT	VP1	2676-2657	6
035	AWITTYTAYGAYGGITGG	VP1	3098-3115	7
036	TAIAIIGTICCCATRTTRTT	VP1	3201-3182	8
040	ATGTAYRTICCIMCIGGIGC	VP1	2951-2970	9
041	GGIGGIGGRTCIGTJAKYTT	VP1	3054-3035	10
045	GAIGARAAYCTIATIGARAC	VP1	2648-2667	11
046	CCCATIAKRTCIATRTCCC	VP1	2820-2801	12
050	GTRCTYACIAIIAGRTCYCT	2A	3513-3494	13
051	TSAARYTGTGCAARGACAC	VP3	2429-2448	14
052	STGYCCAGATTCAGTGT	VP3	2413-2430	15
053	GGNACNCAYRTNATHTGGA	VP3	2216-2235	16
054	GCCITRTTITGRTGICCRAA	2A	3408-3389	17
055	GGIACICAYRTIRTITGGA	VP3	2216-2235	18
187	ACIGCIGYIGARACIGGNCA	VP1	2612-2631	19
188	ACIGCIGTIGARACIGGNG	VP1	2612-2630	20
189	CARGCIGCIGARACIGGNGC	VP1	2612-2631	21
222	CICIGGIGGIAYRWACAT	VP1	2969-2951	22

These primers were designed to amplify a broad range of cDNA fragments drawn from the set of enteroviruses (see Example 2). The primers of SEQ ID NOs:1-22 were designed based on information available regarding known sequences of non-polio enteroviruses, as well as sequences in the VP1 region obtained as part of the development of the present invention (see Example 1; see Table 2 for GenBank accession numbers of the sequences). Additional information used to design the primers of SEQ ID NOs:1-22, especially the primers of SEQ ID NOs:19-22, was obtained from known sequences of other members of the *Picornaviridae* family, as provided in Table 2.

The amplicons obtained from the PCR protocol applied to each prototype virus are sequenced to obtain the nucleotide sequence in each. Procedures that may be used for sequencing include the methods of Maxam and Gilbert (Meth. Enzymol. 65, 499-566 (1980)) and Sanger et al., (Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) (see also Sambrook et al., (1989)). The method of Maxam and Gilbert involves random chemical degradation reactions carried out on a nucleic acid labeled at one end. Each of four separate degradation reactions is specific for a different one of the four bases in the nucleic acid. The method of Sanger et al. involves use of a different 2',3'-dideoxynucleotide chain terminator in each of four template-driven DNA polymerase reactions. The Sanger method is readily implemented in automated sequencing instruments, such as those of PE-Biosystems, Foster City, CA. The VP1 sequences that were obtained with the above procedures were incorporated into the non-polio enterovirus database of the present invention (see Table 2).

Typing of Clinical Isolates Obtained in the Field. A clinical sample is obtained from a subject suspected of harboring an enterovirus. Any suitable clinical specimen may be used for this purpose. Commonly, and by way of nonlimiting example, such a sample may be whole blood or a fraction thereof, a bronchial wash, cerebrospinal fluid, an eye swab, a conjunctival swab, a swab or scraping from a lesion, a nasopharyngeal swab, an oral or buccal swab, pericardial fluid, a rectal swab, serum, sputum, saliva, stool, a stool extract, a throat swab, urine, brain tissue, heart tissue, intestinal tissue, kidney tissue, liver tissue, lung tissue, pancreas tissue, spinal

cord tissue, skin tissue, spleen tissue, thymus tissue, cells from a tissue culture, a supernatant from a tissue culture, or tissue from an experimentally infected animal.

Viral RNA may be isolated from a clinical sample either directly or after inoculating a cell culture with the clinical sample and cultivating a larger virus population. Direct isolation is rapid but may result in low virus titer, whereas inoculation and cell culture will provide a higher titer but may take several days.

In order to obtain amplicons from viral RNA, the RNAs from the virus isolates are treated with a reverse transcriptase primer preparation that contains a random oligonucleotide RT primer, such as a library of random hexanucleotides. The resulting cDNA is amplified in a PCR procedure using a mixture of oligonucleotide primers that hybridize to motifs that are highly conserved throughout the enteroviruses, or more generally, motifs that are highly conserved among the picornaviruses. As used herein, the notion of hybridizing specifically to a highly conserved region encoding a highly conserved amino acid motif relates to identifying at least two nucleotide sequences in the viral genomes which display minimal variation across both the complete spectrum of prototypical enterovirus serotypes, as well as the variants that may be present in clinical samples at any given time. Thus, at least two relatively constant amino acid sequences, or motifs, encoded by these nucleotide sequences, occur phenotypically in all or most of the viruses of the enteroviral species and variants, and the corresponding coding sequences in the nucleic acid are likewise relatively constant across the prototypes and variants. Such conserved or invariant sequences, or motifs, are required in order that a single pair of oligonucleotide primers, or as small a set of such primers as is practical, suffices to prime the amplification of all or the maximum possible number of prototypical viruses and all or the maximum number of viral variants infecting the population at any given time.

In important embodiments of the invention, the primers used are a mixture of oligonucleotides whose use in a PCR amplification provides an amplicon encompassing most or all of the VP1 gene. By way of nonlimiting example, such a mixture may include an oligonucleotide chosen from among an oligonucleotide whose

sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:4, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:9, and a mixture thereof, as well as an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:3 (see Table 3); in particularly important embodiments the oligonucleotides employed according to the above mixtures are primer 011 (SEQ ID NO:3), primer 012 (SEQ ID NO:4), and primer 040 (SEQ ID NO:9). The use of either or both of the primers (012, SEQ ID NO:4 and 040, SEQ ID NO:9) provides specific hybridization to target sequences in the 5' region of the VP1 gene of most or all of the non-polio enteroviruses. The third primer, 011 (SEQ ID NO:3), specifically hybridizes to a target sequence in the 2A region of most or all the non-polio enteroviruses. Each of the primers is disclosed in PCT application WO 98/14611, which is incorporated herein by reference.

More generally, primer sets that include a mixture of oligonucleotides that contain the sequences given by SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, or SEQ ID NO:22 may be employed in amplifying a broad range of picornaviruses. Specifically, oligonucleotides chosen from among an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:19, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:20, an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:21, and mixtures thereof, may be combined with an oligonucleotide whose sequence contains, at the 3' end thereof, the sequence given by SEQ ID NO:22 (see Table 3) for use in the present method. Advantageously, the oligonucleotides included in the above mixtures are primer 187 (SEQ ID NO:19), primer 188 (SEQ ID NO:20), primer 189 (SEQ ID NO:21), and primer 222 (SEQ ID NO:22).

Using the mixtures of oligonucleotide primers set forth in the preceding paragraphs leads to preparation of the enteroviral PCR amplicons according to the method of this invention. The amplicons are then either detected or isolated for sequence analysis. They may be isolated by any of a variety of amplicon purification procedures that serve to provide a purified preparation of the amplicon. These

include, by way of nonlimiting example, gel electrophoresis coupled with visualization using a fluorescent dye and extraction of the detected amplicon from the gel, and extraction from the amplification solution using an immobilized derivative of one or more of the PCR primers to bind a strand of the amplicon after it has been
5 denatured. The purified amplicons may be sequenced using conventional sequencing techniques or procedures.

The nucleotide sequence obtained for the amplicon derived from a particular clinical sample of an enterovirus is then matched with the sequences in the database of prototypical sequences describing the known serotypes of enteroviruses. The
10 sequence matching may be carried out by any suitable sequence matching algorithm designed to determine the extent of identity or similarity between a query sequence in its entirety and a standard or reference sequence. By way of nonlimiting example, such an algorithm may be that of Needleman and Wunsch (J. Mol. Biol. 48:443-453 (1970) implemented in the program Gap in the Wisconsin Sequence Analysis
15 Package, version 9.1), and the like. Such algorithms provide a result that the query sequence most resembles a particular one, and (in most cases) only one, of the reference sequences drawn from the database. According to the present method, the serotype of the enterovirus in the clinical sample is the serotype of the sequence from the database identified as most closely resembling the sequence of the sample.

20 Numerous advantages result upon implementation of the present invention. Typing of an enterovirus in a clinical sample may be done avoiding the necessity of culturing the sample in a cell culture or in a whole animal host (e.g., mouse). Such procedures are cumbersome, labor-intensive and resource-intensive, and pose dangers of infection to the workers conducting the assay. The typing likewise avoids the
25 necessity of conducting a standardized serotyping assay. Serotyping is labor-intensive, and requires the availability of the antiserum pools that are specific or selective for the various enterovirus serotypes. Furthermore, serotyping using these procedures is not very effective because numerous variants and escape mutants in field samples of enteroviruses avoid detection and provide, therefore, a false negative
30 result. The present invention additionally avoids the disadvantages of known PCR

amplification procedures employed with non-polio enteroviruses, which are largely based on the conserved 5' untranslated region of the non-polio enterovirus genome, and thereby lack a means for typing the samples found.

In contrast, the present invention provides the only PCR-based means for typing a clinical sample of an enterovirus available at the present time. The procedure is easy to carry out and provides an unambiguous, and accurate, typing of a clinical sample in a large fraction of test cases that were also typed by standard serotype pools. Typing of cases of enterovirus-caused diseases or syndromes permits an appropriate therapy to be chosen in suitable cases. Such therapy should lead to amelioration of the severity of the disease or syndrome and, hopefully, a complete recovery. Typing furthermore provides important public health and epidemiological information that could lead to protective and/or preventive measures being taken among a population at risk of contracting such a disease or syndrome.

The following examples are intended to illustrate the invention and not to limit it.

Example 1. Establishing a Database of Sequences Corresponding to Standard Non-polio Enterovirus Serotypes. The viruses used for sequence analysis are listed in Table 2, above. The prototypical virus samples were obtained from the American Type Culture Collection. The viruses were propagated in RD cells, HLF cells, LLC-MK₂ cells, or primary monkey kidney cells using Eagle's MEM supplemented with 2% fetal bovine serum or by intracerebral inoculation of newborn mice (see Grandien, M., et al., "Enteroviruses and Reoviruses", in Diagnostic procedures for viral, rickettsial, and chlamydial infections, 6th Ed. (Schmidt, N.J., et al., eds.) 1989, Amer. Public Health Assoc., Washington, DC, pp. 513-578). The isolation of the viral RNA, and the RT-PCR amplification was conducted as described by Oberste et al. (Am. J. Trop. Med. Hyg. 58:41-46 (1998b)). In summary, in this procedure, viral RNA was extracted from infected cell culture supernatants, or from 10% infected mouse brain homogenate with Trizol LS™ (Life Technologies, Inc., Gaithersburg, MD), and cDNA was obtained by use of a set of random hexanucleotide primers (Boehringer Mannheim Biochemicals, Indianapolis, IN), and a SuperScript™

preamplification kit (Life Technologies, Inc.). Reverse transcription was performed in a solution containing 20 mM Tris chloride pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.1 M dithiothreitol, 0.5 mM each of dATP, dCTP, dGTP, and TTP, 0.8 μM random hexamer primer, 5 μL RNA, and 10 U SuperScript II™ reverse transcriptase (Life Technologies, Inc.). The reaction proceeded for 1 h at 42°C.

The resulting cDNAs were amplified by PCR using primers for VP3 and 2A shown in Table 3 (SEQ ID NOs:1-18), in a reaction containing 20 mM Tris chloride pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, and TTP, 1 μM sense-orientation primer, 1 μM antisense-orientation primer 1 μL cDNA from the reverse transcription step, above, and 1.25 U *Thermus aquaticus* DNA polymerase (Life Technologies, Inc.). The reaction was incubated at 94°C for 3 min, then followed by 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 5 min. The specific primer pairs used differed from one virus to another in order to obtain satisfactory yields of the amplicons. For some viruses, VP1 was amplified as two overlapping fragments with internal VP1 primers as well as the VP3 and 2A primers. The PCR products were gel isolated and purified in preparation for sequencing with the QIAquick™ gel extraction kit (QIAGEN, Inc., Santa Clarita, CA), in which DNA is selectively adsorbed to a silica gel membrane at pH below 7.5 at high salt concentration. The impurities are separated from the membrane, then the DNA is eluted therefrom using Tris buffer or water. Sequencing was carried out on an automated DNA sequencer (Applied Biosystems Division, Perkin Elmer, Inc., Foster City, CA) using 2',3'-dideoxynucleotide chain terminators (Sanger et al. (1977)) that carried fluorescent labels.

Complete VP1 PCR products of viruses for which VP1 primers were not available were obtained by cloning the viral cDNA into the plasmid pGEM-T (Promega Corp., Madison, WI). Nested-deletion subclones were constructed from the resulting plasmid with an Erase-a-Base™ kit (Promega Corp.). In this procedure, the plasmid is first digested with a restriction nuclease providing either a blunt end or a 5' overhang. The opened plasmid is then digested with a 3'-5' exonuclease, *E. coli* exonuclease III, to remove plasmid sequences unrelated to the viral VP1 gene. The

extended 5' overhang is then removed using S1 nuclease, and the plasmid is resealed by first repairing the ends with DNA polymerase, then ligating with DNA ligase. The resulting shortened plasmid is propagated in a suitable host to provide larger amounts of the plasmid, including the VP1 sequence. For each virus, at least two independent clones were sequenced by automated methods as described above.

Using these procedures, complete VP1 nucleotide sequences were determined for 57 human non-polio enterovirus strains for which VP1 sequences had not previously been determined. These are summarized in Table 2, which shows both the GenBank accession numbers (numbers AF081293 to AF081349) and the corresponding SEQ ID NOs, 23-79. Forty-seven of the strains were prototype strains for recognized human enterovirus serotypes (Melnick (1996)). The other ten sequenced strains were well-characterized antigenic variants which, while antigenically distinct from their respective prototype strains, were similar enough to them to have been considered to be the same serotype (Committee on Enteroviruses of the National Foundation for Infantile Paralysis, Am. J. Public Health 47:1556-1566 (1957); Melnick (1996)). Combined with the 21 previously available complete enterovirus VP1 sequences, of which 19 are prototypes and 2 are variants, the database constructed for use in the present method includes 66 prototype VP1 sequences and 16 variants or other enteroviruses, including the three poliovirus Sabin strains and the Barty variant of E9.

The boundaries of the newly sequenced VP1 genes were predicted by comparison of the nucleotide and deduced amino acid sequences with those of previously characterized enteroviruses. Human enterovirus VP1 sequences varied in length from 834 to 951 nucleotides (278 to 317 amino acid residues). The CB group has the shortest predicted VP1 amino acid sequences (278 to 298 residues), while EV68 and EV70 had the longest ones (312 and 317 residues, respectively).

Each of the enterovirus VP1 sequences developed in this work is characteristic of the serotype from which it arises, and differs from the sequence of every other serotype. For this reason, the VP1 sequences can be used as markers for the prototypical serotypes of the non-polio enteroviruses. The 66 prototype and 16

variant sequences identified above are used in the method of the present invention to form the content of a database for use in typing an enterovirus obtained in a clinical sample.

5 Example 2. Design of Non-Polio Enterovirus PCR Primers and Assessment of the Breadth of Their Specificity.

10 Design of PCR primers. Since the VP1 sequence was found to correlate with serotype (Example 1), this region was targeted for development of sequence-based molecular diagnostics, namely, generic PCR primers to amplify and sequence a portion of the VP1 gene. Degenerate deoxyinosine-containing PCR primers were
15 designed which specifically recognize regions within or near the termini of the VP1 gene of non-polio enteroviruses. Primers with the broadest specificity within the non-polio enterovirus genus were chosen by searching for regions in the genome that encode amino acid motifs within VP1 and those immediately C-terminal to VP1, in 2A, that are the most conserved across the prototypes. (Echoviruses E22 and E23
20 were excluded, because it is likely that they will be reclassified as members of a new Picornavirus genus, *Parechovirus* (Mayo et al., J. Gen. Virol. 79:649-657 (1997)). The motif MYVPPG (Met-Tyr-Val-Pro-Pro-Gly) was present in the deduced VP1 amino acid sequences of 44 enterovirus prototype strains whose nucleotide sequences are provided in Example 1. Thirteen prototypes had Ile substituted for Val and CA7
25 contained Ala instead of Val. CA12, CA14, and EV71 contain the motif, MFVPPG (Met-Phe-Val-Pro-Pro-Gly). In EV68 and 70, a slightly different motif was present, MYVPTG (Met-Tyr-Val-Pro-Thr-Gly). For viruses in the CB-like phylogenetic group the M(Y/F)(V/I)PPG motif is followed by Gly, whereas in all other enteroviruses, the motif is followed by Ala (A). To account for differences between the virus groups and for codon degeneracy, two different inosine-containing primers
30 were designed to anneal to this region. Primer 012 (ATGTAYGTICCCICGIGG) is based on the amino acid sequence, MYVPPGG (SEQ ID NO:80). Primer 040 (ATGTAYRTICCIMCIGGIGC) is based on the amino acid sequence, MY(V/I)P(P/T)GA (SEQ ID NO:81). The selectivity of these two primers is

primarily due to the first position at the 3' end of each primer (i.e., in primer 012, the base at the 3' end is G, and in primer 040, the base at the 3' end is C) (see Table 3.) In addition, primer 040 contains increased degeneracy at positions 8 and 14 from the 3' end of the primer in order to detect those viruses which encode an isoleucine (position 8) or a threonine (position 14) in these positions. For PCR, primers 012 and 040 were each paired with primer 011 (GCICCGAYTGITGICCRAA), which corresponds to the amino acid motif FG(Q/H)QSGA (Phe-Gly-(Gln/His)-Gln-Ser-Gly-Ala; SEQ ID NO:82), present near the 5' end of the 2A gene and which is conserved among most enteroviruses for which the 2A sequence is available.

10 Specificity of PCR Primers. To assess the breadth of specificity and thereby the general applicability of the 012/011 and 040/011 primer pairs, both pairs were tested in RT-PCR reactions with template RNA derived from each of the human non-polio enterovirus prototype strains (see Figure 2). Primer pair 012/011 amplified 23 of 30 echovirus prototypes (Figure 2C), as well as CA2, CA7, CA9, CA11, CB1, 15 CB2, CB3, CB6, and PV1 (Poliovirus 1) (Figure 2A). Primer pair 040/011 amplified 14 of 23 CA prototypes and PV1 (Figure 2B), as well as E2, E6, E14, E16, E18, E19, E20, E24, E25, E27, E30, and E31 (Figure 2D). Twenty-two prototypes were not amplified by either primer pair (CA10, CA13, CA15, CA16, CA20, CA21, CA22, CB4, CB5, E1, E7, E9, E21, E22, E23, E32, EV68, EV 69, EV70, EV71, as well as 20 PV2 and PV3, where PV signifies poliovirus).

Example 3. Typing of Clinical Isolates Obtained in the Field.

25 Viruses. Fifty-one virus isolates of 24 different serotypes were chosen from those processed in the inventors' laboratory at the Centers for Disease Control and Prevention (CDC) during the period 1991-1998 for routine non-polio enterovirus reference testing. The viruses were from 19 different states in the United States and two other countries, and were chosen to be representative of the serotypes in the collection for the period surveyed. To avoid the effects of sampling bias in the interpretation of sequence comparisons, no more than four isolates of any given

serotype were chosen for sequencing. The isolates included examples of coxsackievirus A, coxsackievirus B, echovirus, and numbered enteroviruses.

Virus isolation and neutralization. The virus strains were isolated from a wide range of clinical specimens, including blood (n=1), cerebrospinal fluid (n=7), conjunctival swab (n=1), "lesion" (n=1), postmortem lung (n=1), nasopharyngeal swab (n=2), sputum (n=1), stool (n=18), throat swab (n=8), and tissue not specified (n=11). Forty-four of the 51 strains were originally isolated by the submitting laboratory, most of which were state public health laboratories in the United States. The remaining seven strains were isolated from original stool specimens at CDC. All isolates were typed antigenically using WHO-standard antiserum pools (Melnick et al., 1973), supplemented with additional pooled and monospecific antisera such that all human enterovirus serotypes, as well as antigenic variants of E4, E6, E11, and E30, could be identified (P. Feorino, personal communication to the inventors).

RNA extraction and RT-PCR. Viral RNA was extracted from infected cell culture supernatant using the QIAamp™ Viral RNA Kit (QIAGEN, Inc.). Reverse-transcription polymerase chain reaction (RT-PCR) was carried out as described previously (Oberste et al., (1998a,b)). From each viral cDNA, an amplicon of approximately 450 bp, encompassing the 3' half of VP1 and the 5' end of 2A, was amplified by PCR using the primers 012/011 or 040/011 (Table 3). Primer specificity was tested by PCR amplification of the prototype strain of each human enterovirus serotype with both primer pairs. Amplification products were visualized by agarose gel electrophoresis and ethidium bromide staining. PCR products from clinical isolates were gel-isolated and purified for sequencing using the QIAquick™ Gel Extraction Kit (QIAGEN, Inc.) and sequenced on an automated DNA sequencer using fluorescent dideoxy-chain terminators as in Example 1 (Applied Biosystems Division, Perkin Elmer, Inc.). The sequences obtained for the clinical samples were deposited in the GenBank sequence database (Accession Numbers AF081595-AF081645).

Sequence analysis. The sequences were compared to the enterovirus VP1 sequence database developed in Example 1 by sequential pairwise alignment of the query sequence with each sequence in the database, using the algorithm of Needleman

and Wunsch (1970), implemented in the program Gap (Wisconsin Sequence Analysis Package, version 9.1). The results of the pairwise comparisons were compiled and sorted in descending order by percent identity with the query sequence.

PCR-amplification of clinical isolates. In order to establish the utility of using viral sequence analysis as an enterovirus typing tool, typing by partial sequencing of VP1 was compared with the conventional serological typing method using 52 clinical isolates typed in the inventors' laboratory from 1991 to 1997. Partial VP1 sequences relate to obtaining sequences in a region of approximately 400 nucleotides at the 3' end of the VP1 gene. Despite the failure of primer pair 012/011 to amplify the E7, E9, E21, CB4 and CB5 prototype strains (see Example 2), 012/011 successfully amplified recent clinical isolates of each these serotypes. Likewise, primer pair 040/011 amplified recent isolates of CA16, CA21, and EV71, but not the prototype strains of these serotypes (see Example 2). Taken together, these two primer pairs failed to amplify only one clinical isolate of the 52 tested, a 1993 EV6 isolate from Texas (TX93-1673). The presence of amplifiable RNA in the latter specimen was confirmed by amplification of 5'-specific sequences by pan-enterovirus primers (data not shown). For the other 51 isolates, a VP1-specific fragment was amplified from purified RNA by RT-PCR using primer pairs 012/011 or 040/011. In most cases, only one of the two primer pairs produced an amplicon of the expected size (data not shown).

Typing of clinical isolates by nucleotide sequence analysis. The PCR products were gel isolated and sequenced. The sequences were compared to the complete enterovirus VP1 database developed in Example 1 by pairwise alignment of the isolate sequence to each sequence in the database using the program Gap. These comparisons produced, for each clinical isolate, a set of values of the percent identity giving the extent of identity between the sequence of the given clinical isolate and each of the prototype sequences in the database. Typing was obtained as that prototype whose extent of identity to the clinical sample was the highest of all the prototypes. In general, as implemented in this study, if the highest global identity is >75%, the clinical sample and the prototype are of the same serotype. If the highest score is 70%-75%, the identification is presumptive and should be confirmed by

neutralization using monospecific antisera specific for each of the four highest scoring prototypes. If the highest score is <70%, the clinical sample is considered to be of no known serotype; for example, it may be from a picornavirus for which a sequence is not yet available, or it may be a new enterovirus serotype. For each clinical isolate,

5 the matches with the highest and second highest pairwise identity score were identified. Table 4 shows the serotype as obtained from the classical neutralization test, as well as the types of the highest and next highest scoring prototypes obtained in this way (with entries giving the extent of identity of both the nucleotide sequences (nt) and the translated amino acid sequences(aa)). Strains in Table 4 are identified by

10 U.S. state (two letter code) or country (three letter code) of origin, year of isolation, and lab identifier number. For example, WA91-0374 indicates that the strain was isolated in the state of Washington in 1991 and the lab sample number was 0374. The abbreviations DOR and PER in Table 4 designate the Dominican Republic and Peru, respectively.

Table 4. Correspondence Between Typing by Sequence and by Neutralization.

Strain	Neut. Type	Highest Scoring Prototype			Second Highest Scoring Prototype(s)			
		Type	nt (%)	aa (%)	Type	nt (%)	Type	aa (%)
WA91-0374	E6	E6	83.3	95.6	E1	69.7	E29	74.3
OR91-1426	E30	L30	85.8	92.9	E21	69.5	E21	81.7
CT92-1465	E16	E16	81.4	93.6	E5	72.2	E5	78.6
FL92-1512	CB2	CB2	86.5	98.5	CB4	68.3	CB4	75.2
WA92-1516	E11'	E11	77.1	90.1	E11	72.9	E19	83.0
NC92-1612	E9	E9	77.8	94.6	E17	70.2	E16	72.9
GA92-1616	E11	E11	77.6	89.4	E19	72.2	E19	82.3
TX92-1647	CA14	CA14	86.8	91.1	CA7	63.4	CA7	67.9
MD92-1649	E25	E25	77.1	91.5	E1	68.5	E21	77.6
DOR93-1657	CA24v	CA24	77.4	92.8	CA20	67.6	CA17	75.9
FL93-1763	E11'	E11	78.5	90.1	E19	72.6	E19	83.0
GA93-1763	CA9	CA9	93.8	95.3	E4	68.6	E4	70.8
GA93-1765	E7	E7	79.7	95.7	E32	68.8	E32	77.1
M093-1808	E25	E25	77.6	91.5	E33	67.5	E21	76.9
ME93-1814	CB5	CB5	95.2	98.5	CB1	71.3	CB1	77.7
NM93-1816	CB3	CB3	90.3	97.7	CB6	69.9	CB1	81.5
OR93-1817	E25	E25	77.9	91.5	E1	68.5	E21	76.9
WA93-1821	E4	E4	81.1	96.1	E1	73.1	E1	80.9
MN94-1828	E25	E25	76.9	92.2	E29	67.9	E21	77.6
WA94-1849	E3	E3	79.6	93.0	E7	68.2	E12	80.0
AR94-1884	E30	E30	96.0	93.6	E21	70.0	E21	82.4
GA93-2460	CB5	CB5	95.8	93.5	CB1	70.8	CB1	77.7
GA93-1892	E30	E30	85.5	93.6	E21	69.5	E21	83.4
GA93-1994	E7	E7	79.7	95.7	E32	69.1	E32	77.1
NM94-1919	EV71	EV71	80.6	93.4	CA16	66.9	CA16	76.6
AZ94-1925	CA14	CA14	86.5	97.0	CA7	63.8	CA7	68.2
RI94-1959	E21	E21	78.3	93.7	E30	69.6	E30	80.0
CT94-2006	EV71	EV71	80.3	93.4	CA16	66.0	CA16	76.6

Strain	N ut. Type	Highest Scoring Prototype			Sec nd Highest Scoring Prot type(s)			
		Type	nt (%)	aa (%)	Type	nt (%)	Type	aa (%)
MD95-2037	EV71	EV71	79.9	92.7	CA16	67.0	CA16	76.6
AZ94-2060	CA21	CA21	90.9	98.6	CA24	68.7	CA24	75.5
PA94-5753	CA16	CA16	77.9	94.7	EV71	68.7	EV71	83.0
NM95-2070	E6	E6	76.8	94.1	E29	68.1	E29	75.5
TX95-2089	E13	E13	72.4	88.7	EV69	71.5	EV69	93.0
GA95-2093	CA21	CA21	91.4	98.6	CA24	67.5	CA24	75.5
GA95-2095	CA16	CA16	77.9	94.9	EV71	69.4	EV71	77.4
NC95-2135	CB2	CB2	83.2	99.2	CB4	68.3	CB4	76.2
AR95-2139	E9	E9	75.7	92.8	E17	70.0	E1	71.8
TX95-2147	CA16	CA16	76.5	94.9	EV71	70.4	EV71	77.4
VA95-2154	E11'	E11	78.3	90.8	E19	71.7	E19	83.7
WT95-7151	E9	E9	75.7	93.5	E17	69.4	E16	71.4
VA95-2157	E30	E30	85.3	92.1	E21	70.0	E21	82.1
GA96-2175	CA9	CA9	81.5	92.6	E19	68.4	E11	72.3
CT96-2181	E5	E5	86.5	92.9	E31	71.5	E31	82.1
CT96-2181	E18	E18	75.7	93.6	E17	69.9	E4	75.4
TX96-2184	CA21	CA21	91.6	98.6	CA24	68.2	CA24	75.5
TX97-2320	E18	E18	78.8	92.9	E17	69.7	E17	74.5
NH97-2342	CB3	CB3	77.4	98.5	CB5	67.9	CB1	84.6
PER98-2528	E6	E6	86.0	95.6	CB1	71.6	E29	74.3
PER98-2533	E7	E7	80.4	95.7	E32	68.1	E12	78.6
PER98-2537	E11	E11	78.5	94.3	E19	71.9	E19	82.3
PER98-2558	E33	E33	79.3	96.9	CB1	70.3	E4	75.4

The typing results for the 51 isolates shown in Table 4, fully correlate with the serotype as determined by the conventional neutralization test (Table 4). The nucleotide sequences of the various clinical isolates ranged from 72.4% identity to 95.2% identity with the sequences of the respective prototype strains and only from 63.4% identity to 73.1% identity to the sequences of the second highest scoring

prototypes. The predicted amino acid sequences of the clinical isolates ranged from 88.7% identity to 98.5% identity with that of the cognate prototype strain and from 67.7% identity to 84.6% identity to that of the second highest scoring prototype strain. With one exception, the difference between percent nucleotide sequence identity to the highest scoring prototype and the percent identity to the second highest scoring prototype was 4.2%. In the exception (TX95-2089), typed antigenically as E13, the highest-to-second-highest difference was only 0.9% (72.4% identical to E13 vs. 71.5% identical to EV69), suggesting that either TX95-2089 has diverged significantly from E13 or EV69, or that the E13 prototype strain (Del Carmen) is not representative of the serotype as a whole. When the complete VP1 nucleotide sequence of TX95-2089 was examined, it was found to be 72.6% identical to that of the E13 prototype, 70.1% identical to that of the EV69 prototype (second highest score), and 64.7% identical to that of the E12 prototype (third highest score). The predicted complete VP1 amino acid sequence of TX95-2089 was 88.2% identical to that of E13, 80.8% identical to that of EV69 (second highest score), and 70.0% identical to that of CB1 (third highest score), suggesting that TX95-2089 is probably a strain of E13 which has diverged in nucleotide sequence by accumulating mutations in the third codon position. TX95-2089 was neutralized by monospecific anti-E13 antisera but not by monospecific anti-EV69 antisera (data not shown).

The typing procedure described in this invention contravenes the evaluation of the state of the art in Holland et al. (J. Clin. Microbiol. 36:1588-1594 (1998)), which states that PCR is not able successfully to type enterovirus infections. Furthermore, Oberste et al. (1998a) conducted sequence and phylogenetic analyses of all human enterovirus serotypes based on a portion of the VP2 gene. They determined that this portion of VP2 may be inappropriate for consistent molecular inference of serotype. For these reasons, the method of the present invention, as described above and exemplified in Examples 1-3, provides results that are unexpected by workers in the field.

Example 4. Detection of a Broad Range of Picornaviruses.

The present method has been applied to the detection of a broad range of picornaviruses that afflict both human and nonhuman subjects, according to the procedures generally followed in Example 2.

5 In addition to the primers 011, 012, and 040, additional primers directed to the detection of human and nonhuman picornaviruses were devised. These are provided as Primer 187 (ACIGCIGYIGARACIGGNCA) (SEQ ID NO:19) that hybridizes to a sequence encoding the amino acid motif TA(A/V)ETGH (SEQ ID NO:83), Primer 188 (ACIGCIGTIGARACIGGNG) (SEQ ID NO:20) that hybridizes to a sequence
10 encoding the amino acid motif TAVETG(A/V) (SEQ ID NO:84), Primer 189 (CARGCIGCIGARACIGGNGC) (SEQ ID NO:21) that hybridizes to a sequence encoding the amino acid motif QAAETGA (SEQ ID NO:85), and Primer 222 (CICCIGGIGGIA YRWACAT) (SEQ ID NO:22) that hybridizes to a sequence encoding a motif M(F/Y)(I/V)PPG(A/G) (SEQ ID NO:86) (see Table 3). Primer 187
15 is directed to amplification of the CB and E groups in the forward direction (i.e., it hybridizes to the sense strand of the cDNA), Primer 188 is directed to amplification of the poliovirus (PV) group, EV68 and EV70 in the forward direction, Primer 189 is directed to amplification of the group of CA16-like viruses (Oberste et al., J. Virol. 73:1941-1948 (1999)) in the forward direction, and Primer 222 is directed to
20 amplification of all enteroviruses in the reverse direction (i.e., it hybridizes to the antisense strand of the cDNA).

In this example, prototypical serotypes of human enteroviruses were subjected to RT-PCR using, in separate experiments, primer pairs 012/011 (SEQ ID NOs:3 and 4), 040/011 (SEQ ID NOs:3 and 9), 187/222 (SEQ ID NOs:19 and 22), 188/222 (SEQ
25 ID NOs:20 and 22), and 189/222 (SEQ ID NOs:21 and 22). The results are shown in Table 5. Additionally several serotypes from a selection of human and nonhuman picornaviruses, namely bovine enterovirus, human rhinovirus, and simian picornavirus, were examined according to the present method. For simian picornaviruses and HRV2, actual experiments were done. For the other serotypes
30 considered, provision of an amplicon was predicted by comparison of the primer

sequences to each of the viral VP1 sequences. The results of this experiment are shown in Table 6.

Table 5. Amplification of Human Enterovirus Serotypes by Specific Primer Pairs.

Virus	012/011	040/011	187/222	188/222	189/222
CA1	-	-	-	■	□
CA2	□	■	□	□*	■
CA3	-	■	-	□	■
CA4	-	■	-	-	■
CA5	-	■	□	□*	■
CA6	-	■	-	□*	■*
CA7	-	-	±	-	■
CA8	-	□	-	□	■
CA9	■	-	■*	□	-
CA10	-	-	-	□	■
CA11	-	±	-	■	□
CA12	-	■	-	□*	■
CA13	-	-	□*	■	□
CA14	-	■	-	□	■
CA15	-	-	□	■	□
CA16	-	■	-	-	■
CA17	-	±	±	■	□
CA18	-	■	-	(±)	-
CA19	-	±	-	■	□
CA20	-	-	-	■	±
CA21	-	■	-	■	□
CA22	-	-	-	■	□
CA24	-	■	-	■	□
CB1	■	-	■	-	-
CB2	■	-	■	□*	±
CB3	■	±	■*	-	±
CB4	-	-	■*	-	±
CB5	■	-	■	□	□
CB6	■	-	■	□*	□*
PV1	-	■	□	■	□
PV2	-	-	□	■	□*
PV3	-	-	-	■	□
E1	-	-	■	-	-
E2	■	□	■	-	±

Virus	012/011	040/011	187/222	188/222	189/222
E3	■	—	■	—	±
E4	■	—	■*	□	□*
E5	■	—	■	—	±
E6	■	□	■	—	±
E7	■	—	(±)	—	□
E9	■	—	■	—	±
E11	■	—	■*	—	±
E12	■	—	■*	—	□*
E13	■	—	■	—	□
E14	■	□	■	—	□*
E15	—	—	■	—	—
E16	■	—	■	—	±
E17	■	—	■*	—	±
E18	■	□	■	□	□
E19	■	—	■	—	±
E20	■	□	■	□	±
E21	■	—	■	—	—
E24	■	□	■	—	±
E25	■	□	■	—	±
E26	■	—	■	—	±
E27	■	□	■*	—	±
E29	—	—	■	—	—
E30	■	□	■	—	±
E31	■	□	■*	—	±
E32	—	—	■	—	±
E33	■	—	■	—	—
EV68	—	—	□	■	□
EV69	—	—	■	—	—
EV70	—	—	—	■	□
EV71	—	■	—	—	■

CA, coxsackie A virus; CB, coxsackie B virus; PV, poliovirus; E, echovirus; EV, numbered enterovirus. Results are for amplification of prototype strains and/or clinical isolates of the indicated serotypes, based on testing in a standard RT-PCR assay for human enteroviruses (Oberste et al., 1999).

□ and ■ : strong amplification, single band on gel; ■ indicates the primer pair giving optimal amplification for a particular serotype.

± and (±) : weak amplification, single band on gel; (±) indicates the primer pair giving optimal amplification for a particular serotype.

□* and ■* : strong amplification, multiple bands on gel; ■* indicates the primer pair giving optimal amplification for a particular serotype.
 - : No amplification observed.

Table 6. Predicted and Observed Results of Amplification of Picornavirus Serotypes by Specific Primer Pairs.

Virus	012/011	040/011	187/222	188/222	189/222
BEV1				■	
BEV2a				■	
BEV2b				■	
HRV1b			■		
HRV2			■		
HRV3				■	
HRV14				■	
HRV16			■		
HRV89			[(±)]		
SPV2		■			
SPV9	-	-	-	-	-
SPV10		■			
SPV11	-	-	-	■	-
SPV12	-	-	-	-	■
SPV13		■			
SPV15	-	-	-	■	-
SPV16	-	-	-	-	■
SPV17			■		□

BEV, bovine enteroviruses; HRV, human rhinovirus; SPV, simian picornavirus.
 Results are for amplification of prototype strains and/or clinical isolates of the indicated serotypes, based on testing in a standard RT-PCR assay (Oberste et al., 1999) for HRV2, and simian picornaviruses. For the other viruses (indicated by square brackets []), the entry provides a predicted result based on comparison of the primer sequences with the available VP1 nucleotide sequences found in the GenBank database.

□ and ■ : strong amplification, single band on gel; ■ indicates the primer pair giving optimal amplification for a particular serotype.

(±) : weak amplification, single band on gel, optimal amplification for a particular serotype.

- : No amplification observed.

Empty cells indicate primer-template combinations that have not yet been tested.

The results for 012/011 and 040/011 in Table 5 tabulate the observations already discussed with respect to Figure 2 in Example 2.

5 Taking the results for primer pairs 187/222, 188/222, and 189/222 in Tables 5 and 6 together, it is seen that these primer pairs amplify all human enteroviruses, and five of the six simian picornaviruses tested. They should also amplify the three bovine enteroviruses and all six human rhinoviruses for which VP1 sequences are available in GenBank; other than HRV2, these have not yet been directly tested. Furthermore, the three simian picornaviruses that were not tested using primer pairs 187/222, 188/222, and 189/222 were successfully amplified by primer pair 040/011
10 (see Table 6).